

REMARKS / ARGUMENTS

The Final Office Action mailed November 27 2006 has been received and reviewed. Applicants respectfully request reconsideration of the application in view of the following remarks and arguments.

CLAIM AMENDMENT

Claims 21-37 are pending in the application. Claims 21-28, which are currently under consideration, read upon isolated protein complexes; whereas, claims 29-37, which are all ultimately dependent upon claim 21, but are presently withdrawn, read upon methods of using such isolated protein complexes. As previously noted, claims 21-28 are related to claims 29-37 as “product and process of using that product.” Hence, should claim 21 be found to be in condition for allowance, Applicants respectfully request rejoinder of claims 23-37, in accordance with the provisions outlined in M.P.E.P. § 821.04(b).

Of all claims currently under consideration, only claim 25 is being amended. The amendment is necessary to correct an obvious error that was discovered during the preparation of this response. Specifically, the word “second,” in lines 2 & 3 of the claim, is being replaced with the word “first,” since the claim refers to a protein that “comprises an amino acid sequence that is at least 80% identical to amino acid residues 304 to 471 of PRAK” that interacts with ERK3, and such a protein would clearly be categorized as a “first protein” described in part (ii) of claim 21.

Applicants submit that this amendment should be entered into the record in order to place Claim 25 in condition for allowance, or, in the alternative, in better condition for appeal. Further, Applicants assert that Examiner should enter the amendment because it introduces no new matter into the application, and does not require any further search.

PRIORITY

The Final Office Action continues to allege that the present application may not claim priority to U.S. Application Nos. 60/168,377 (“the ‘377 Application”) and 09/727,384 (“the ‘384 Application”) for detectable, in vitro PRAK:ERK3 complexes. More specifically, the Final Office Action alleges that neither priority document discloses

“critical conditions,” such as those on page 200 – 201 of the instant Specification, for the formation of an in vitro complex from isolated PRAK and ERK3 components. Final Office Action, p. 3, first paragraph. Applicants respectfully disagree.

The ‘384 Application discloses, through a valid incorporation by reference, examples of specific conditions for the formation and detection of isolated protein complexes. U.S. Patents Nos. 5,622,852 (“the ‘852 Patent”) and 5,773,218 (“the ‘218 Patent”) and PCT published application WO97/27296 (“the PCT Application”) are incorporated into the ‘384 Application by reference as teaching specific detection techniques. ‘384 Application, p. 20, ll. 24 – 28. The ‘852 Patent itself describes the use of affinity chromatography and immunoprecipitation to detect an isolated protein complex in great detail (including sample reaction conditions and reagents). ‘852 Patent, col. 33, ll. 10 – 55; col. 58, l. 50 – col. 59, l. 7; col. 44, ll. 31 – 54. Such methods are well-known in the art, mirror those disclosed on pp. 200 – 201 of the instant Specification, and, far from being specific to the interaction between Bad and bcl-2, may easily be applied by a skilled artisan to virtually any protein complex. Id. at col. 33, ll. 35 – 38 (labeled Bad protein may be contacted with immobilized bcl-2, bcl-x², or Bad polypeptides); see also, ‘218 Patent, col. 27, ll. 10 – 60.

In sum, the isolation of a protein complex in vitro is mundane in the art and, notwithstanding this routine nature, Applicant’s disclosed isolation and detection techniques would be quite sufficient for one skilled in the art to isolate a PRAK:ERK3 complex in vitro. In view of these teachings of the ‘377 Application, Applicants respectfully submit that the priority date for the detectable in vitro formation of a PRAK:ERK3 complex should be December 2, 1999 – the filing date of the ‘377 Application.

THE REJECTIONS

Provisional Double Patenting Rejection

The Final Office Action reaffirms the following provisional, nonstatutory, obviousness-type, double patenting rejections, presumably under MPEP § 804(II)(B) (Rev. 5, Aug. 2006, pp. 800-21 – 800-26):

1. Claims 21, 23, 24, 27 and 28 remain provisionally rejected as being unpatentable over Claim 1 of co-pending Application no. 10/035,344.
2. Claims 21, 23, 24, 27 and 28 remain provisionally rejected as being unpatentable over Claims 1 – 3 of co-pending Application no. 10/194,385.
3. Claims 21, 23, 24, 27 and 28 remain provisionally rejected as being unpatentable over Claims 21 and 30 of co-pending Application no. 10/267,476.

This provisional rejection is reaffirmed based on the allegations (1) the rejected claims “state no lower limit on the size of a fragment,” and (2) the Specification contains a “non-structural definition of ‘interaction’ at page 11,” (3) the rejected claims “lack descriptions of a particular structure for a PRAK or ERK3 kinase involved in an interaction,” (4) the rejected claims do not require that the claimed polypeptides “share substantial structural relationship to a particular amino acid sequence region of either of SEQ IDs NOS:730 or 732.” Final Office Action, paragraph bridging pp. 4 – 5.

The first allegation is not problematic because it is inaccurate in practice. It is true that the claims state no explicit lower limit on the size of the fragments involved in the claimed complexes. The claims are limited, however, to fragments that are capable of interacting. See, e.g., Claim 21. This functional limitation guarantees only fragments of a sufficient length to interact will be encompassed by the claims. See Invitrogen Corp. v. Clontech Laboratories, Inc., 429 F.3d 1052 (Fed. Cir. 2005) (hereinafter “Invitrogen”) (declaring valid a claim to a modified polynucleotide without any explicit lower limit on the size of the modified polynucleotides encompassed by the claims).

As to the second allegation, the Final Office Action fails to explain both what is meant by a “non-structural definition of ‘interaction’” and what bearing this has on the nonstatutory obviousness-type double patenting rejection. The definition of “interaction” in the Specification simply restates the meaning given by those skilled in the art. The Final Office Action goes on to allege that the Claims require “neither a particular structure nor a particular interaction.” Final Office Action, p. 5. This is simply untrue. Claim 21 requires that any polypeptide have the ability to interact with another specific polypeptide (e.g., ERK3 fragments must be able to interact with PRAK). Furthermore, Claim 21 requires a “particular structure” when it limits interacting polypeptides to full-length PRAK (or ERK3), a fragment thereof, a homologue of either the full-length

protein or its interacting fragments, or a fusion protein containing one of these. In other words, all polypeptides encompassed by the claims share structural similarities with native PRAK or ERK3 of the kind exemplified in Invitrogen and the Written Description Guidelines.

Finally, Applicants question how the last two allegations at all bear on the allowance of the instant claims. This ground of rejection appears to be, at its core, based on the allegation that kinases generally share some conserved regions of similarity. Applicants respectfully point out, however, that this rejection must fail because no Office Action has satisfied its prima facie burden by presenting evidence of sequence similarity between the proteins of the instant and copending applications. Even if such evidence were presented, in order to have any force the rejection would need to further present evidence that kinases in the copending applications show sequence similarity to PRAK and ERK3 outside of the kinase domains. No such evidence has been offered.

Different protein kinases do indeed share stretches of similarity. However, most of the similarity between different kinases is found in their kinase domains. New, *et al.*, PRAK, a novel protein kinase regulated by the p38 MAP kinase, *EMBO J.* 17:3372-3384, Figures 1 & 2, pp. 3373 & 3374 (June 1998); provided herewith in its entirety as *Exhibit A*. PRAK, for example, contains eleven conserved kinase domain motifs and shows some sequence similarity to three other protein kinases. *Id.* (comparing PRAK to MAPKAP-K2, MNK1 and the C-terminal kinase domain of RSK2). This similarity and the conserved kinase domains are almost completely limited, however, to regions outside of the region shown to interact with ERK3. *Id.*; see also, Specification, p. 18, Table 1. Applicants respectfully submit that no prima facie evidence has been presented that the kinase domains of PRAK are at all responsible for its interaction with ERK3. By extension, there is no evidence that other unidentified kinases would show PRAK's interaction capabilities because there is no evidence of a protein that shows sequence similarity within the interaction domain reported in Table 1.

Claim Rejections under 35 USC § 112, 1st paragraph, Written Description

Claims 21 – 28 stand finally rejected under 35 USC § 112, first paragraph as being based upon a disclosure that allegedly lacks sufficient written description of the

invention. The Final Office Action attempts to distinguish between the instant application on the one hand, and Example 14 of the Written Description Training Materials and Invitrogen v. Clontech on the other. See generally, USPTO Written Description Training Materials (hereinafter “Training Materials”), available at <http://www.uspto.gov/web/menu/written.pdf> (last visited January 15, 2007); Invitrogen, 429 F.3d 1052. Applicants respectfully submit that not one meaningful distinction exists.

Regarding Example 14 of the Written Description Training Materials (hereinafter “Example 14”), the Final Office Action alleges the present claims

are drawn to isolated complexes comprising members of two broad genera of kinase fragments and variants having no particular assayable characteristics, formed under no particular conditions, while the specification provides a written description of only one isolated complex formed under conditions set forth at page 200 of the specification by a specific fragment of a specific PRAK kinase and a specific fragment of a specific ERK3 kinase.

See Final Office Action, p. 7, first paragraph. Applicants initially note that at least two of these points of alleged distinction are plainly incorrect. First, the Office Action emphasizes that the instant claims include no limitation as to the conditions under which the complex is formed. Example 14, however, says nothing about the conditions under which the reaction of A to B takes place. Second, the Office Action alleges that “none of the rejected claims requires that either a divergent PRAK or a divergent ERK3 have a particular activity that is amenable to an assay for that activity disclosed in the specification.” With all due respect, Applicants find this allegation utterly inexplicable given (1) the specific limitations in the claims that all polypeptides encompassed thereby must interact with another specific polypeptide and (2) Applicants’ exhaustive disclosure of interaction-detecting assays [See Specification, p. 11, ll. 19 – 28; p. 98, ll. 17 – 29; p. 116, l. 4 – p. 123, l. 12 (describing several different assays available for detecting interaction); p. 196, l. 3 – p. 197, l. 12 (describing the yeast two-hybrid assay in great detail)].

Another “distinction” that is anything but a distinction is found in the Office Action’s emphasis on the fact that Applicants have disclosed “only one isolated complex” between one specific PRAK fragment and one specific ERK3 fragment. This is actually

a point of identity between Example 14 and the present case because Example 14 specifically states “[t]here is a single species disclosed, that species being SEQ ID NO: 3 [representing the full length, unmodified protein].” Written Description Training Materials, p. 54, second paragraph. This is only the beginning of the striking similarities, however. Example 14 states that “[t]he procedures for making variants of SEQ ID NO: 3 are conventional in the art and an assay is described which will identify other proteins having the claimed catalytic activity.” Written Description Training Materials, p. 53, third paragraph. Making variants of SEQ ID NO:730 or SEQ ID NO:732 of the present invention (or variants of the nucleic acids encoding them) is no less conventional and in fact involves the exact same procedures. See Specification, p. 14 l. 1 – p. 15, l. 13 (defining “homologue” and giving examples of their production and detection); p. 100, ll. 18 – 20 (stating that “various nucleotide substitutions, deletions or insertions can be incorporated into the nucleic acid molecule by standard molecular biology techniques”). Multiple assays for detecting interaction activity are disclosed in the Specification, as mentioned above.

Likewise, there is no meaningful distinction between the instant case and Invitrogen. The Office Action attempts to distinguish the claim at issue in Invitrogen based on the following alleged differences: (1) the modified reverse transcriptase polypeptide has “characteristics that could be determined by two separate assays disclosed in the patent specification;” (2) the patent specification discloses the “particular structural alteration” of “deletion of one domain;” and (3) the patent specification discloses structural modifications that would stabilize a modified product of the patent claim.” See Final Office Action, paragraph bridging pp. 7 – 8.

Applicants initially point out that “stabilizing structural modifications” are not recited in Claim 1 of the patent at issue in Invitrogen. See U.S. Pat. No. 6,063,608, Claim 1 (hereinafter “608 Patent”). Regardless, Applicants disclosed at least one means for stabilizing the claimed protein complexes. Specification, p. 99, ll. 8 – 24 (detailing a method of stabilizing a protein complex including cross-linking the complex members). Additionally, as discussed at length above, Applicants have disclosed several different means for determining the interaction characteristic. Id. at p. 11, ll. 19 – 28 (describing several different assays for detecting protein-protein interactions, including protein

affinity chromatography and immunoprecipitation); p. 98, ll. 17 – 29; p. 196, l. 3 – p. 197, l. 12 (describing the yeast two-hybrid assay in great detail).

More importantly, the Office Action alleges Applicants have provided

no guidance as to where to make structural changes ... throughout the amino acid sequences of the specific, integral PRAK and ERK3 kinase fragments indicated in Table 1 at page 18 that will provide products the activity of which can be assayed according to the specification.

Final Office Action, p. 8, first paragraph. This statement of the alleged problem contains its own solution. Applicants have provided specific guidance as to which portions of the full-length PRAK or ERK3 proteins may be modified (as by substitution or, in the specific working example of the disclosure, deletion) while retaining interaction function. See Specification, p. 18, Table 1 (listing the PRAK interaction domain as consisting of, at most, amino acid residues 304 and 471 and the ERK3 interaction domain as consisting of, at most, amino acid residues 36 and 502). In addition, Applicants have described a method for further refining the interaction domain starting with the narrowing guidance already disclosed in Table 1 of the Specification. Id. at p. 98, ll. 17 – 29.

While the '608 Patent specification indeed discusses making deletions from the wild-type reverse transcriptase (RT) enzyme in order to arrive at a protein that has RT activity but substantially reduced RNase H activity, at no time does the specification structurally (e.g., by sequence) define any specific "domain" that is responsible for the undesirable RNase H activity or put any specific structural limit on how many or which residues may be deleted. Instead, the specification places a functional limitation on which modifications may be made while remaining within the scope of the claims (i.e. retains RT activity but has substantially reduced RNase H activity). Applicants likewise have not put any structural limit (other than the percentage identity for homologues) on which specific amino acids may be deleted or modified, but have instead placed a functional limit on such modifications (i.e. retains ability to specifically interact).

Regarding guidance, the '608 Patent specification states that "[l]ittle is known about the structure-functional relationship of these [RT and RNase H] activities" ('608 Patent at col. 1, ll. 28 – 2) and the most "guidance" given is that "deletion of deoxyribonucleotides at the 3' end of the gene which encode the portion of the

polypeptide having RNase H activity" will yield an enzyme that lacks RNase H activity. '608 Patent at col. 10, l. 58 – col. 11, l. 15 (then stating that this end-wise deletion method yielded a modified gene consisting of approximately nucleotides 1018 to 2512 and encoding a protein of 508 amino acids with RT activity and substantially no RNase H activity). The present Specification need not specifically describe which residues within the already narrowed fragments of PRAK and ERK 3 may be modified or deleted while retaining interaction activity under Invitrogen because the '608 Patent specification does not detail which residues within the 508 amino acids of the example polypeptide may be modified while retaining the desired catalytic properties. In short, Applicants have provided at least as much guidance as the patentee in Invitrogen.

When considered together, therefore, Example 14 and Invitrogen collectively clamor for withdrawal of the instant rejection. Applicants' Claims and Specification meet all the requirements of Example 14 by disclosing at least one species example and by claiming interactions involving polypeptides defined in both structural and functional terms. Applicants have exceeded that required by Invitrogen by requiring that modified interaction polypeptides (i.e. homologues of PRAK, ERK3 and their fragments) exhibit at least 75% identity to the art-known wild-types. As the presently claimed invention is sufficiently described in light of Example 14 and Invitrogen, Applicants respectfully request that the rejection based on 35 U.S.C. § 112, first paragraph, Written Description be withdrawn.

Claim Rejections under 35 USC § 112, 1st paragraph, Enablement

Claims 21 – 28 stand finally rejected under 35 USC § 112, first paragraph as being based upon a disclosure that allegedly provides insufficient enablement for the full scope of the claims. Specifically, the Final Office Action alleges that the claims are not enabled because (1) they encompass "myriad combinations" of polypeptides, as small as four amino acids, with no guidance on which modifications may be made in the amino acid sequences, Final Office Action, p. 9 – 10; (2) "the definition of 'interact' in the specification requires no particular, assayable interaction," id. at 9, last paragraph; and (3) the Specification teaches isolation and detection of "specific, not generic" PRAK and ERK3 components, id. Applicants respectfully disagree.

Enablement is lacking only if one skilled in the art would be unable to practice the invention without engaging in undue experimentation. In re Wands, 858 F.2d 731, 736-737 (Fed. Cir. 1988). “A considerable amount of experimentation is permissible, if it is merely routine, or if the specification [gives] a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.” Id. at 737 (emphasis added). Thus an applicant need only show either that the experimentation required is routine or that the specification gives reasonable guidance, but not both. As will be shown, Appellant has amply met both of the above alternative requirements in the present case, thus going beyond that which is required under In re Wands.

In re Wands is the benchmark in biotech enablement analysis. At issue was monoclonal antibody technology, specifically assays using high-affinity monoclonal antibodies to hepatitis B-surface antigen (HBsAg). Id. at 734. The Board contended claims to such assays were not enabled because screening for the high-affinity antibodies required undue experimentation. Id. at 739. More specifically, the Board pointed to what it deemed a very low success rate, which in turn meant artisans would need to undertake undue experimentation to find antibodies with the desired binding affinity. Id.

The Federal Circuit reversed the Board, however, holding that the invention could be practiced without undue experimentation. Id. at 740. The court noted that the applicant’s success rate was higher than the Board contended, but more importantly the court emphasized the routine nature of the experimentation needed to obtain antibodies within the scope of the claim. Id. Even a laborious process comprising “immunizing animals, fusing lymphocytes from the immunized animals with myeloma cells to make hybridomas, cloning the hybridomas, and screening the antibodies produced by the hybridomas for the desired characteristics” did not, in the court’s estimation, represent an “excessive” amount of effort. Id. The court also found helpful the “considerable guidance” in the specification, guidance that consisted solely of instructions on how to carry out the above experiment steps. See generally, U.S. Patent No. 4,879,219 (‘219 patent), which derives from the application at issue in In re Wands.

The present case is analogous to Wands in that no undue experimentation is needed because the experimentation called for is purely routine. Much like in Wands, several experimental steps must be carried out in order to find protein complexes that fall

within Applicants' claims additional to those actual examples disclosed in the Specification. See Specification, p. 104, l. 24 – p. 109, l. 29 (describing production of protein complexes); p. 108, ll. 22 – p. 109, l. 13 (specifically discussing production of protein fragments and homologues); p. 196, l. 3 – p. 197, l. 12, p. 140, l. 1 – p. 143, l. 18 (describing yeast-two hybrid system and its use in screening for interaction or disruption of interaction between protein fragments). All of these steps, however, are routine within the art. At the time of filing, for example, it was a routine task for a skilled artisan to identify proteins orthologous to PRAK or ERK3, and to align such proteins with PRAK or ERK3 in order to identify regions of conservation. Amino acid changes outside the regions of conservation would less likely result in loss of interacting capability. The Final Office Action has not met the burden of showing, and indeed has only raised conclusory allegations, that these steps are anything other than routine. The enablement inquiry could end here.

Just as in Wands, however, Applicants have given ample guidance on how to carry out these routine experiments. See Specification, p. 104, l. 24 – p. 109, l. 29 (describing production of protein complexes); p. 108, ll. 22 – p. 109, l. 13 (specifically discussing production of protein fragments and homologues); p. 196, l. 3 – p. 197, l. 12, p. 140, l. 1 – p. 143, l. 18 (describing yeast-two hybrid system and its use in screening for interaction or disruption of interaction between protein fragments).

In fact, Applicants have exceeded that required by Wands by describing the screening process in detail and providing specific guidance regarding which protein homologues and fragment homologues might form complexes. The Wands application gives no guidance on which of the thousands of cells extracted from an immunized mouse will ultimately yield anti-HBsAg antibodies with sufficient affinity to satisfy the elements of the claim. See generally, '219 patent. Instead the application simply offers instructions and examples on how to carry out the arduous screening process needed to find high-affinity IgMs. Id. Applicants have gone further, giving artisans a starting point for finding interacting homologues and fragments by indicating probable binding regions of both PRAK and ERK3. Specification, p. 18, Table 1; p. 98, ll. 17 – 29. With all due respect, the Final Office Action has given no reasoned basis for doubting that this guidance represents at least a "reasonable amount of guidance with respect to the

direction in which the experimentation should proceed.” Wands, 858 F.2d at 737 (emphasis added).

Thus, given the Specification’s description of art-routine experiments and its directions regarding which amino acid positions might be tolerant of substitution, one of ordinary skill in the art would know how to (1) perform alignments of amino acid sequences of homologous proteins, (2) identify conserved amino acid residues, (3) introduce conservative changes to specific residues in regions of proteins not exhibiting significant conservation, and (4) test whether the engineered variant proteins are still capable of interacting.

As discussed in a previous section, the Final Office Action’s allegation that the claims may encompass small polypeptides is unproblematic. The claims are limited, however, to fragments that are capable of interacting. See, e.g., Claim 21. **This functional limitation guarantees only fragments of a sufficient length to interact will be encompassed by the claims.** See Invitrogen, 429 F.3d 1052 (declaring valid a claim to a modified polynucleotide without any explicit lower limit on the size of the modified polynucleotides encompassed by the claims).

In sum, while it is true that extended time and effort might be required to identify all possible PRAK and ERK3 fragments and homologues within the scope of the claims, “the mere fact that the experimentation may have been difficult and time consuming does not mandate a conclusion that such experimentation would have been considered to be ‘undue’ in the art.” Falkner v. Inglis, 448 F.3d 1357, 1365 (Fed. Cir. 2006) (quoting Board Op.).

As noted in the previous section, the allegation that “the definition of ‘interact’ in the specification requires no particular, assayable interaction” is without basis in view of (1) the specific limitations in the claims that all polypeptides encompassed thereby must interact with another specific polypeptide and (2) Applicants’ exhaustive disclosure of interaction-detecting assays. See Specification, p. 11, ll. 19 – 28; p. 98, ll. 17 – 29; p. 116, l. 4 – p. 123, l. 12 (describing several different assays available for detecting interaction); p. 196, l. 3 – p. 197, l. 12 (describing the yeast two-hybrid assay in great detail).

Finally, the Final Office Action apparently alleges that ELISA methods, which are very well-known in the art, would not be suitable to isolate in vitro complexes between PRAK homologues or fragments and ERK3 homologues or fragments, even though ELISA methods may be used to isolate in vitro complexes comprising full-length PRAK and ERK3. See Final Office Action, p. 9; Specification, p. 199, l. 13 – p. 201, l. 10 (describing ELISA). Once again, no evidence is presented and only a conclusory allegation is made. Careful consideration of the nature of ELISAs and the language of Claim 21, however, requires the opposite conclusion.

The ELISA test described in the Specification detects a complex between a PRAK fusion protein and an ERK3 fusion protein. The polypeptide region fused to ERK3 (GST) is responsible for fixing the fusion protein to the wells of the ELISA plate. Specification, p. 200, ll. 8 – 11. The Final Office Action has offered no evidence that GST is unable to perform this vital function simply because it is fused to a fragment or homologue of ERK3 that falls within the scope of Claim 21. The polypeptide fused to PRAK (myc) is responsible for reporting the presence of a complex. Id. at p. 201, ll. 1 – 10 (anti-myc antibodies are added to the well after time has been given for the PRAK and ERK3 portions of each fusion to interact). The Final Office Action has offered no evidence that myc is unable to perform this vital function simply because it is fused to a fragment or homologue of PRAK that falls within the scope of Claim 21. Finally, the key to the assay is the interaction between ERK3 and PRAK, which produces the complex and allows the anti-myc antibodies to report the interaction after washing. By definition, fragments, homologues, and homologues of fragments of ERK3 that fall within the scope of Claim 21 are capable of interacting with PRAK. Thus, no evidence has been offered that even calls into question whether fragments or homologues encompassed by Claim 21 may be isolated in vitro. Most importantly, because these ELISAs are extremely routine in the art and may be done 96 or more at a time, there is no evidence that one skilled in the art would be unable to isolate such a complex in vitro without undue experimentation.

In view of the above arguments, Applicants submit that the as-filed Specification provides sufficient enablement to support the full scope of the claimed invention, and therefore meets the requirement of 35 U.S.C. § 112, first paragraph. Consequently,

Applicants respectfully request that the enablement rejection of Claims 21 – 28 be withdrawn.

CONCLUSION

Applicants believe that once the arguments presented above are considered, the outstanding objection and rejections will be withdrawn, and the pending claims will be in condition for allowance. Should the Examiner determine that additional issues remain which might be resolved by a telephone conference, he is invited to contact the undersigned via his direct line (801-883-3463).

It is believed that no extension of time, or any additional fees are due with this response. If this is incorrect, an extension of time as deemed necessary is hereby requested, and the Commissioner is hereby authorized to charge any appropriate fees, or credit any over payment, to Deposit Account no. **50-1627**.

Respectfully submitted,

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PRAK, a novel protein kinase regulated by the p38 MAP kinase

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We have identified and cloned a novel serine/threonine kinase, p38-regulated/activated protein kinase (PRAK). PRAK is a 471 amino acid protein with 20–30% sequence identity to the known MAP kinase-regulated protein kinases RSK1/2/3, MNK1/2 and MAPKAP-K2/3. PRAK was found to be expressed in all human tissues and cell lines examined. In HeLa cells, PRAK was activated in response to cellular stress and proinflammatory cytokines. PRAK activity was regulated by p38 α and p38 β both *in vitro* and *in vivo* and Thr182 was shown to be the regulatory phosphorylation site. Activated PRAK in turn phosphorylated small heat shock protein 27 (HSP27) at the physiologically relevant sites. An in-gel kinase assay demonstrated that PRAK is a major stress-activated kinase that can phosphorylate small heat shock protein, suggesting a potential role for PRAK in mediating stress-induced HSP27 phosphorylation *in vivo*.

Keywords: HSP27/MAP kinase/p38/PRAK/protein kinase

Introduction

A variety of extracellular stimuli produce cellular responses via activation of the mitogen-activated protein (MAP) kinase cascades (Davis, 1993; Waskiewicz and Cooper, 1995; Su and Karin, 1996; Fanger *et al.*, 1997; Robinson and Cobb, 1997). The specificity of the cellular response is determined by the activation of a particular MAP kinase pathway in response to a given stimulus and by the activation of downstream targets by a given MAP kinase. MAP kinase family members have been found to regulate diverse biological functions by phosphorylation of specific target molecules found within the cell membrane, cytoplasm and nucleus, and thereby participate in the regulation of a variety of cellular processes including cell proliferation, differentiation and immune responses (Blenis, 1993; Marshall, 1994; Cano and Mahadevan, 1995; Seger and Krebs, 1995).

p38 (or p38 α , also known as CSBP and RK) is a MAP kinase superfamily member which was first identified and cloned as an intracellular protein rapidly tyrosine

phosphorylated upon treatment of macrophages with lipopolysaccharides (Han *et al.*, 1993, 1994). p38 α was also cloned as a specific target of pyridinyl imidazole derivatives such as SB203580 which inhibit the production of proinflammatory cytokines by monocytes (Lee *et al.*, 1994). Three closely related kinases of p38 subsequently were cloned and characterized: p38 β (Jiang *et al.*, 1996), p38 γ (also known as ERK6 or SAPK3) (Lechner *et al.*, 1996; Li *et al.*, 1996; Mertens *et al.*, 1996) and p38 δ (also known as SAPK4) (Goedert *et al.*, 1997; Jiang *et al.*, 1997; Wang *et al.*, 1997). p38 α and p38 β are sensitive to SB203580 inhibition, but the activities of p38 γ and p38 δ are unaffected (Goedert *et al.*, 1997). The entire family of p38 MAP kinases can be activated by osmotic changes in the extracellular environment. Other stimuli, such as UV light, oxidation and proinflammatory cytokines, are potent activators of specific p38 family members (Raingeaud *et al.*, 1995; Jiang *et al.*, 1997). In addition, some growth factors, such as erythropoietin and interleukin-3 (IL-3), are activators of p38 α in certain cell types (Foltz *et al.*, 1997; Nagata *et al.*, 1997). All four p38 family members are activated by MAP kinase kinase (MKK) 3 or MKK6 via phosphorylation of the TGY motif (Derijard *et al.*, 1995). Several proteins have been identified as substrates of p38 α including transcription factors (Price *et al.*, 1996; Wang and Ron, 1996; Han *et al.*, 1997; Janknecht and Hunter, 1997; Whitmarsh *et al.*, 1997) and protein kinases (Rouse *et al.*, 1994; Ludwig *et al.*, 1996; McLaughlin *et al.*, 1996; Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997).

Protein kinase substrates of MAP kinases are believed to play an important role in amplifying and diversifying MAP kinase signals. Three groups of kinases have been shown to be activated by MAP kinases, ribosomal S6 kinase (RSK also known as MAPKAP-K1), MAP kinase-activated protein kinase (MAPKAP-K) and MAP kinase-interacting kinase or MAP kinase signal-integrating kinase (MNK) (Rouse *et al.*, 1994; Zhao *et al.*, 1996; Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997). The activity of RSK proteins is regulated by the ERK kinase pathway, and these proteins play important roles in a variety of processes including cell proliferation (Jones *et al.*, 1988; Blenis, 1993; Zhao *et al.*, 1996). The two known MAPKAP-K family members, MAPKAP-K2 and MAPKAP-K3, can be activated by p38 α *in vitro* and their activation can be inhibited *in vivo* by SB203580, a specific inhibitor of p38 α and p38 β (McLaughlin *et al.*, 1996). In turn, activated MAPKAP-K2/3 can phosphorylate small heat shock protein 27 (HSP27) (Stokoe *et al.*, 1992b), lymphocyte-specific protein 1 (LSP1) (Huang *et al.*, 1997a), cAMP response element-binding protein (CREB), ATF1 (Tan *et al.*, 1996) and tyrosine hydroxylase (Thomas *et al.*, 1997). A third group of MAP kinase-regulated protein kinases, MNK, was identified recently (Fukunaga and

Fig. 1. The deduced amino acid and nucleotide sequence of human PRAK. The 11 protein kinase subdomains are indicated under the protein sequence with Roman numerals. The potential phosphorylation site (Thr182) of PRAK is marked by a solid triangle. The asterisk indicates a stop codon. The cDNA sequence of PRAK has been deposited in the DDBJ/EMBL/GenBank database under the accession number AF032437.

Hunter, 1997; Waskiewicz *et al.*, 1997). These kinases are regulated by p38 and ERK, but not by JNK. MNK1/2 can phosphorylate eukaryotic initiation factor-4E (eIF-4E) *in vitro*, which suggests an important link between MAP kinase activation and translational initiation (Waskiewicz *et al.*, 1997).

Despite the identification of several protein kinases as substrates for p38, there is evidence of additional downstream protein kinases. For example, Iordanov *et al.* reported that short wavelength UV irradiation (UVC) elicits p38-dependent CREB phosphorylation, and that the phosphorylation was mediated by an unknown p38-activated protein kinase (Iordanov *et al.*, 1997). To understand better the regulation and function of the p38 pathways, it is necessary to identify and characterize substrates of p38. Here, we describe the structure, function and regulation of a new protein kinase, p38-regulated/activated kinase (PRAK). This enzyme is activated by

stress-related extracellular stimuli. The regulatory phosphorylation site of PRAK is Thr182 and this residue is phosphorylated by p38 α and p38 β *in vitro*. Activated PRAK specifically phosphorylates HSP27 at its physiologically relevant sites *in vitro*. In-gel kinase assays indicated that PRAK is one of the major stress-activated HSP27 kinases in whole-cell extracts from HeLa cells, suggesting that PRAK has a potential role in mediating stress-induced small heat shock protein phosphorylation *in vivo*.

Results

Molecular cloning of human PRAK

Although the RSK and MAPKAP-K share only 35% amino acid sequence identity, a common feature of these MAP kinase-regulated kinases was identified. Each contains the phosphorylation site LX*TP located within the

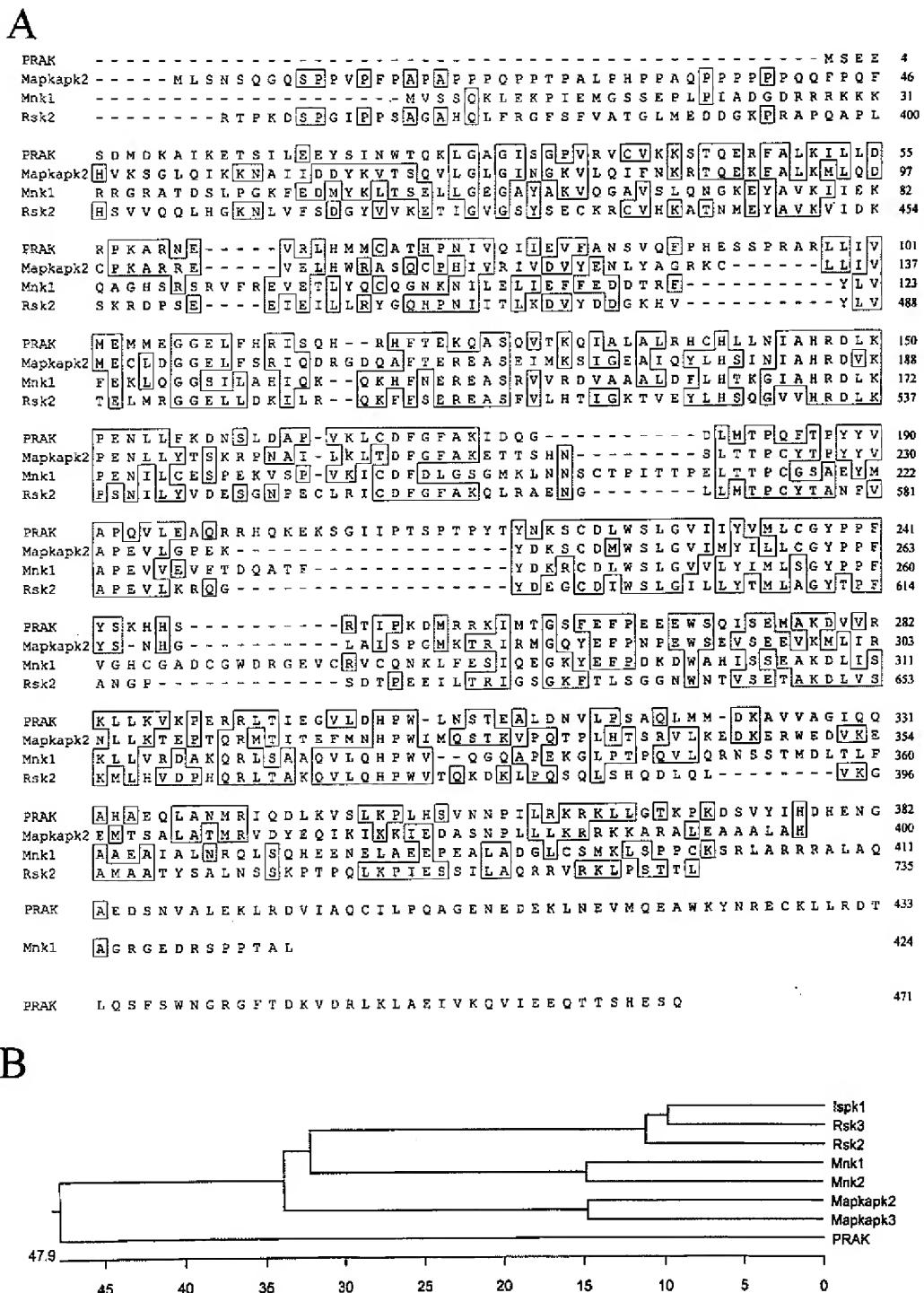


Fig. 2. (A) Sequence comparison of human PRAK, MAPKAP-K2, MNK1 and the C-terminal kinase domain of RSK2 using the Lasergene Megalign program (DNAStar, Madison, WI). Residues conserved between two or more protein kinases are boxed. (B) The ancestral relationships among human PRAK, human MAPKAP-K2, human MAPKAP-K3, human MNK1, murine MNK2, human RSK1 (JSPK1), human RSK2, and human RSK3 are presented as a phylogenetic tree created by the Lasergene Megalign program. The scale beneath the tree measures the distance between sequences, and units indicate substitution events.

T-loop (also called Loop 12) between kinase domains VII and VIII. The sequences adjacent to this phosphorylation site are also conserved within the RSK and MAPKAP-K proteins. We hypothesized that there may be additional MAP kinase-regulated protein kinases that contain con-

served LXTP sites and distinct flanking sequences. The peptide sequences LXTPCYTPYYVAP and LXTPCY-TANFVAP from MAPKAP-K and RSK respectively were used to search the dbest database using the tblastn program. Seven expressed sequence tag (EST) clones were identified

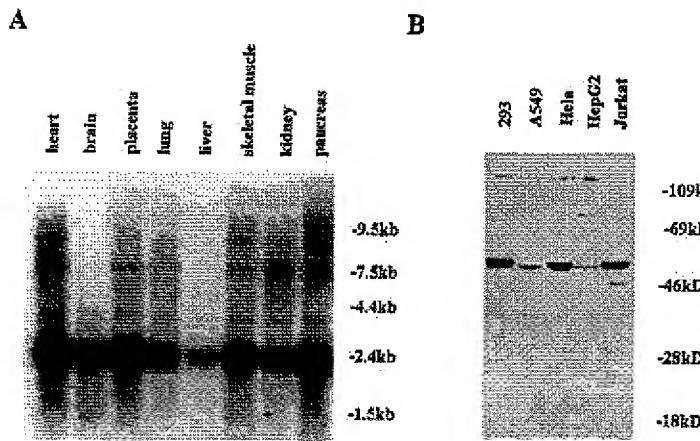


Fig. 3. Expression of PRAK. (A) A blot containing 2 μ g of poly(A)⁺ RNA isolated from various human tissues was hybridized with a probe specific to PRAK. (B) Equal amounts of cell-free lysates from 293, A549, HeLa, HepG2 and Jurkat cells were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane. PRAK was detected using a specific polyclonal antibody.

that contained the LXTP site and novel flanking sequences. The sequence LTTPCGSAEYMAP was present in four EST clones (DDBJ/EMBL/GenBank accession Nos AA080236, W24753, AA083972 and AA157440). The sequence LXTPQFTPYYVAP was found in a single EST clone (DDBJ/EMBL/GenBank accession No. R68329) and the sequence LKTPCFTLHYAAP was present in two clones (DDBJ/EMBL/GenBank accession Nos N57096 and H09985). Additional sequences of these EST clones indicated that they are cDNAs for novel protein kinases. We hypothesized that these clones represent three new protein kinase groups that are regulated by MAP kinases. The EST clones of DDBJ/EMBL/GenBank accession Nos AA157440, R68329 and N57096 were sequenced completely and used to screen a human placental cDNA library to isolate full-length cDNA clones. While this study was in progress, two new protein kinases were reported by Waskiewicz *et al.* (1997) and Fukunaga and Hunter (1997), termed MNK1 and 2, that contained the sequence LXTPCGSAEYMAP. These protein kinases appear to belong to a new protein kinase group because they have only 30% identity to the RSK and MAPKAP-K. Importantly, this group of protein kinases is regulated by MAP kinases as our hypothesis would predict (Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997). Because the available evidence suggests our approach may identify new kinase substrates of MAP kinases, we focused our efforts on the cDNA clones isolated from the human placental cDNA library that encode a LXTPQFTPYYVAP sequence. Complete sequencing of the three isolated clones revealed that they all encoded a single protein. The nucleotide sequence of the longest clone is shown in Figure 1. No in-frame stop codon was found at the 5' end of the clone. 5'-RACE was performed but no additional 5' sequence was obtained. Therefore, we tentatively assigned the first ATG as the start codon. The cDNA has an open reading frame encoding a protein of 471 amino acids with a calculated molecular mass of 54 kDa (Figure 1). This is consistent with the molecular mass of the endogenous protein on Western blots (Figure 3, see below). Since this protein kinase is regulated/activated specifically by p38 (see below), we named this kinase p38-regulated/activated kinase (PRAK). PRAK contains the conserved

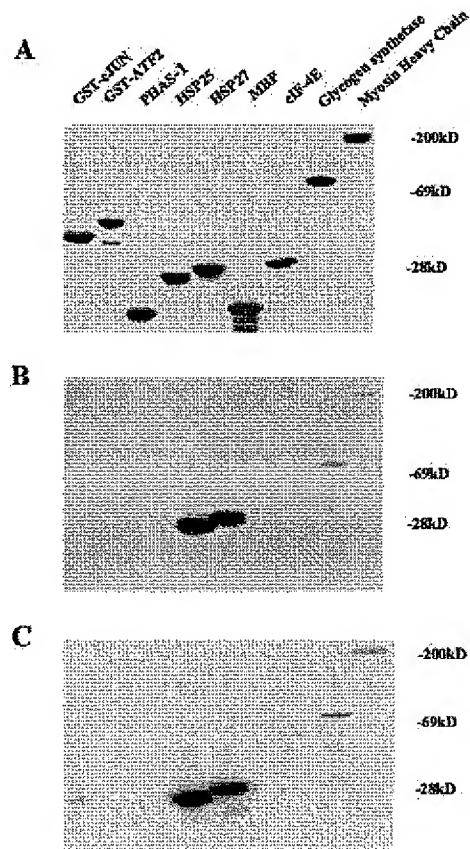


Fig. 4. HSP25 and HSP27 are preferred substrates for PRAK *in vitro*. (A) The Coomassie Blue stain of the protein substrates used in the kinase assays. Approximately 10 μ g of GST-c-Jun (1-93), GST-ATF2 (1-109), PHAS-1, HSP25, HSP27, MBP, eIF-4E, glycogen synthetase and myosin heavy chain were used as substrates, and 0.5 μ g of fully activated PRAK (B) or MAPKAP-K2 (C) were used as kinases for *in vitro* kinase assays. The kinase reactions were stopped by adding SDS sample buffer, and reaction products were analyzed by SDS-PAGE. Substrate phosphorylation was detected by autoradiography, and was quantified by phosphoimaging. Comparable results were obtained in two independent experiments.

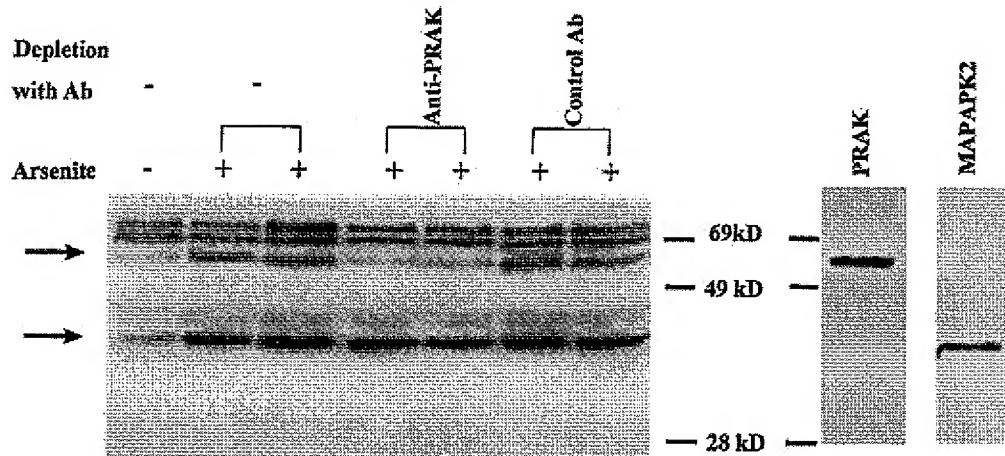


Fig. 5. PRAK is a HSP27 kinase activated by arsenite treatment in HeLa cells. In-gel kinase assay using HSP27 polymerized inside SDS-PAGE. The cell lysates of HeLa cells treated with or without 200 μ M arsenite for 30 min were analyzed. Depletion of PRAK in the cell lysate was achieved by using anti-PRAK polyclonal antibody. The positions of PRAK and MAPKAP-K2 were determined by Western blotting as shown in the right hand panel.

protein kinase domains I–XI which are characteristic of all protein kinases (Figure 1). A DDBJ/EMBL/GenBank database search revealed that PRAK is most closely related to the MAPKAP-K, MNK and RSK kinases. A sequence alignment of PRAK with a member of the RSK group, MAPKAP-K group and MNK group proteins is shown in Figure 2A. PRAK is 32.8, 33.1, 22.8, 25.4, 22.7, 20.0 and 20.1% identical to MAPKAP-K2, MAPKAP-K3, MNK1, MNK2, ISPK1 (RSK1), RSK2 and RSK3, respectively. A computer-generated diagram showing the relatedness of these protein kinases is shown in Figure 2B. The relatively low homology of this protein to the known MAP kinase-regulated protein kinases supports our prediction that the protein containing LXTPQFTPYYVAP may belong to a new kinase group. The tissue distribution of PRAK mRNA was assessed by Northern blot hybridization (Figure 3A). The PRAK transcript is ~2.4 kb and is present in all eight tissues examined (Figure 3A). PRAK protein expression was analyzed in five different human cell lines by Western blot using an anti-PRAK antibody. The protein was detected in all of the cell lines as a 54 kDa protein, consistent with the predicted molecular weight (Figure 3B).

HSP27 is a preferred substrate for PRAK in vitro

To identify the substrate of PRAK, we tested a panel of proteins which are known substrates of PRAK-related protein kinases. As shown in Figure 4B, HSP27 and HSP25 are good substrates of PRAK *in vitro*. Phosphorylation of HSP27 and HSP25 by PRAK is similar in magnitude to that seen with MAPKAP-K2 when equal amounts of fully activated kinase were used in *in vitro* kinase assays (compare Figure 4B and C). Both PRAK and MAPKAP-K2 can phosphorylate glycogen synthetase, and myosin heavy chain to a lesser extent. PHAS-1, MBP, eIF-4E and the N-terminal portions of c-Jun (1–93) and ATF2 (1–109) were poor substrates for PRAK and MAPKAP-K2. To determine if PRAK is a major kinase for HSP27 in stress-activated cells, an in-gel kinase assay using recombinant HSP27 as substrate was performed (Figure 5, right panel). There are four major kinases in HeLa cell

lysates that phosphorylate HSP27. Stimulation of HeLa cells with arsenite resulted in activation of the 45 and 54 kDa HSP27 kinases. Depletion of PRAK in cell lysates with an anti-PRAK polyclonal antibody specifically removed the kinase activity associated with the 54 kDa protein, while the other HSP27 kinases were unaffected. These data strongly suggested that PRAK was a major stress-activated kinase that can phosphorylate HSP27. Western blot analysis (Figure 5, left panel), revealed that the stress-activated 45 kDa HSP27 kinase in HeLa cell lysates is MAPKAP-K2. MAPKAP-K2 has been reported to phosphorylate serines 15, 78 and 82 of HSP27 in response to stress or mitogens *in vivo* (Stokoe *et al.*, 1992b; Knauf *et al.*, 1994). Therefore, we performed an experiment to determine whether or not PRAK phosphorylates HSP27 at the same sites as MAPKAP-K2. Recombinant HSP27 treated with PRAK or MAPKAP-K2 in the presence of [γ -³²P]ATP was used for tryptic phosphopeptide mapping. As shown in Figure 6, PRAK and MAPKAP-K2 phosphorylated the same peptides. Thus PRAK, like MAPKAP-K2, phosphorylates HSP27 at the functionally relevant sites. The overlapping specificity of the two kinases, MAPKAP-K2 and PRAK, is not without precedent (Whitmarsh *et al.*, 1995; Price *et al.*, 1996; Janknecht and Hunter, 1997). However, the physiological relevance of this overlap requires further investigation.

PRAK is activated by environmental stress and proinflammatory cytokines

A variety of agonists were tested for their ability to activate endogenous PRAK in HeLa cells. PRAK activity was measured using an immunokinase assay with HSP27 as the substrate. A Western blot demonstrated that equal amounts of PRAK were used in each assay (data not shown). As shown in Figure 7A, PRAK was strongly activated by stress stimuli such as arsenite, anisomycin and H₂O₂, and by the proinflammatory cytokine tumor necrosis factor- α (TNF- α). Phorbol-12-myristate-13-acetate (PMA) and calcium ionophore A23187 also activated PRAK but to a lesser extent. In contrast, IL-6 had no effect on PRAK activation in HeLa cells. Growth-

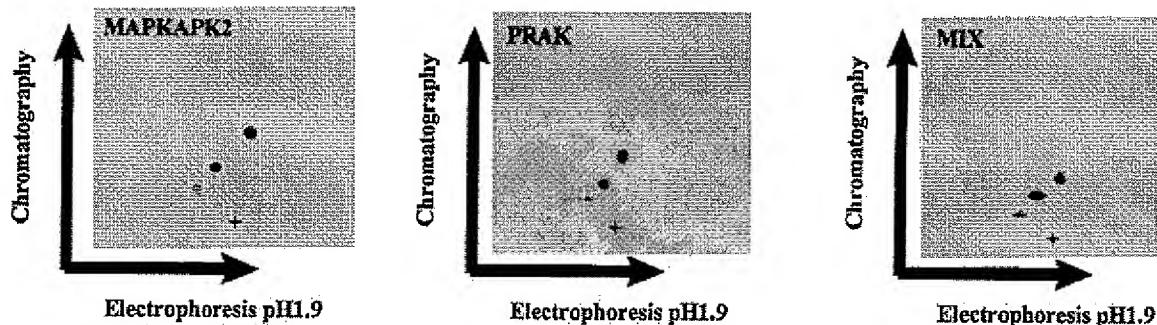


Fig. 6. Tryptic phosphopeptide map of HSP27 phosphorylated by activated GST-PRAK and GST-MAPKAP-K2 *in vitro*. The panel marked as MIX indicates a mixture of equal amounts of HSP27 proteins phosphorylated by GST-PRAK and GST-MAPKAP-K2 prior to tryptic phosphopeptide mapping. The + sign on each panel indicates the original sample loading site. The perpendicular arrowheads indicate two-dimensional separations of phosphopeptides by electrophoresis and chromatography.

related stimuli epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and serum had no or only modest effects on PRAK activity in HeLa cells (Figure 7B). A similar activation profile was obtained when transiently expressed hemagglutinin (HA)-tagged PRAK was assayed by immunokinase assay using anti-HA monoclonal antibody 12CA5 (data not shown).

PRAK is a physiological substrate for p38 α and p38 β

PRAK was cloned because it contained a putative phosphorylation site for MAP kinases. The data presented above demonstrate that PRAK is activated by a panel of stimuli that are known to activate p38 and c-Jun N-terminal kinase [JNK also known as stress-activated protein kinase (SAPK)]. We therefore hypothesized that PRAK is a downstream target of the p38 or JNK signaling pathways. To test this hypothesis, we performed an *in vitro* kinase assay using recombinant PRAK as the substrate and extracellular signal-regulated kinase 2 (ERK2), JNK2, p38 α , p38 β , p38 γ or p38 δ as the kinase (Figure 8). Equal amounts of each recombinant MAP kinase, activated by the appropriate upstream kinase (see Materials and methods), were used (Figure 8A). p38 α , p38 β , p38 γ and p38 δ showed higher activity toward PRAK in comparison with JNK2 and ERK2 (Figure 8B). Coupled kinase assays were performed to address whether the phosphorylation of PRAK by these MAP kinases influenced intrinsic PRAK activity using HSP27 as the substrate. PRAK activity was dramatically increased when it was phosphorylated by p38 α or p38 β (Figure 8C). In contrast, the enhancement of PRAK activity by p38 γ , p38 δ , JNK2 or ERK2 was not significant. Interestingly, p38 δ efficiently phosphorylated PRAK (Figure 8B), but did not lead to activation of PRAK activity (Figure 8C). The inability of p38 δ to activate PRAK is probably due to its failure to phosphorylate the regulatory site(s) of PRAK (see below).

Next, we determined if PRAK was downstream of the p38 pathway in intact cells. Substitution of the phosphorylation sites of MKK with glutamic acid (E) or aspartic acid (D) results in constitutively active forms of MKKs. Previously, we reported that expression of constitutively active MKK by recombinant adenovirus could activate a given MAP kinase pathway in intact cells (Huang *et al.*,

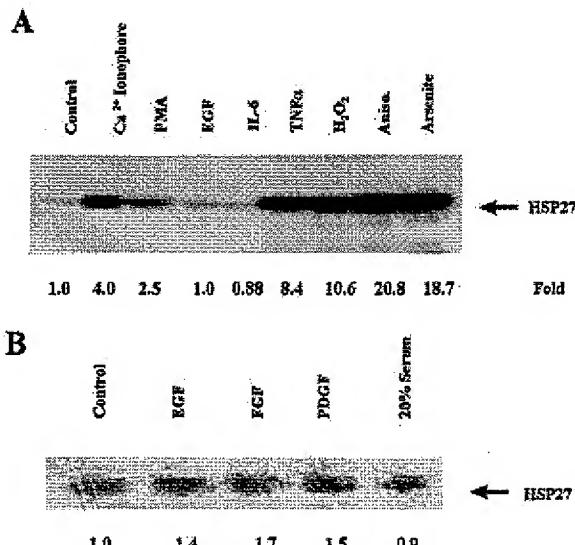


Fig. 7. Activation of endogenous PRAK by various stimuli. HeLa cells were cultured in DMEM with 10% FBS. Approximately 10^6 cells (per sample) were treated with the Ca²⁺ ionophore A23187 (5 μ M), PMA (100 nM), EGF (1 ng/ml), IL-6 (250 U/ml), TNF- α (100 ng/ml), H₂O₂ (400 μ M), anisomycin (50 ng/ml), arsenite (200 μ M) (A), or EGF (1 ng/ml), bFGF (10 ng/ml), PDGF-BB (10 ng/ml) and 20% serum (B) for 20 min at 37°C. Cells were serum starved for 5 h before the stimulation with growth factors. The kinase activity of PRAK isolated by immunoprecipitation with anti-PRAK antiserum was measured using HSP27 as the substrate. The fold activation by the different stimuli was calculated by dividing the radioactive intensity of phosphorylated HSP27 for each treatment by the untreated control and is shown under each lane. Similar results were obtained in two experiments.

1997b). Here, the same strategy was used independently to activate the p38, JNK or ERK pathway by expression of MKK1(E), MKK6(E) or MKK7(D). Twenty four hours after cells were infected with recombinant viruses, endogenous PRAK activity was measured using an immunokinase assay (Figure 9). Expression of MKK6(E), which activates the p38 signaling pathway, caused endogenous p38 α / β and PRAK activation, supporting the concept that PRAK is regulated by the p38 pathway. In contrast, MKK7(D) expression activated JNK1 but not PRAK, and expression of MKK1(E) enhanced ERK2 activity but had

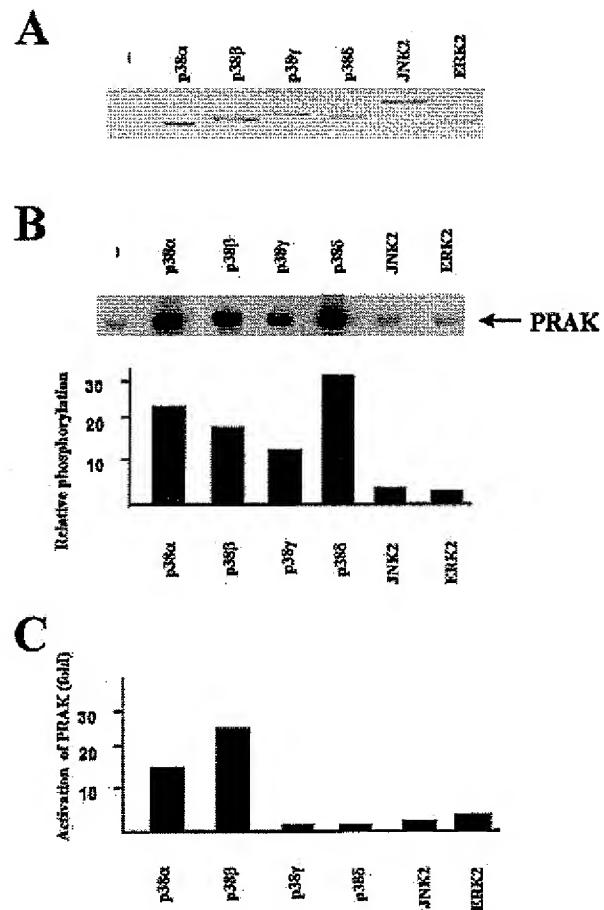


Fig. 8. Phosphorylation and activation of PRAK by different MAP kinases *in vitro*. (A) Coomassie Blue stain of recombinant MAP kinases used in the experiments. (B) Phosphorylation of PRAK (5 μ g) by different MAP kinases (0.5 μ g) that were activated *in vitro* by MKKs as described in Materials and methods. The phosphorylated products were resolved by SDS-PAGE and quantified by phosphoimaging. The relative phosphorylation of PRAK by each MAP kinase was calculated by subtracting the intensity of PRAK autophosphorylation from that of each MAP kinase-treated PRAK. The result is shown as a bar graph. (C) Activation of PRAK by different MAP kinases was assessed by *in vitro* coupled kinase assays. Several paired kinase reactions, containing equal amounts of each MAP kinase (0.5 μ g) and 10 μ g of HSP27 in the presence or absence of 2 μ g of GST-PRAK, were performed. The phosphorylated products were resolved by SDS-PAGE and quantified by phosphoimaging. The fold activation of PRAK by p38 α , p38 β , p38 γ , p38 δ , JNK2 and ERK2 was determined by the formula: [(radioactive intensity of HSP27 phosphorylated by a given MAP kinase activated PRAK and this MAP kinase) – (radioactive intensity of HSP27 phosphorylated by the given MAP kinase alone)]/[radioactive intensity of HSP27 phosphorylated by untreated PRAK]. Comparable results were obtained in two independent experiments.

no influence on PRAK activity. Therefore, JNK1 and ERK2 are unable to activate PRAK *in vivo*. Since MKK1 and MKK7 activate all ERK and JNK isoforms (Cowley *et al.*, 1994; Marshall, 1994; Holland *et al.*, 1997; Lu *et al.*, 1997; Tournier *et al.*, 1997), these data suggest that ERK1 and JNK2/3 are also unable to activate PRAK. Thus, PRAK is regulated specifically by the p38 pathway in intact cells.

To test further whether PRAK is regulated by p38 α / β under physiological conditions, arsenite, TNF- α and PMA were used to stimulate HeLa cells that had been pre-

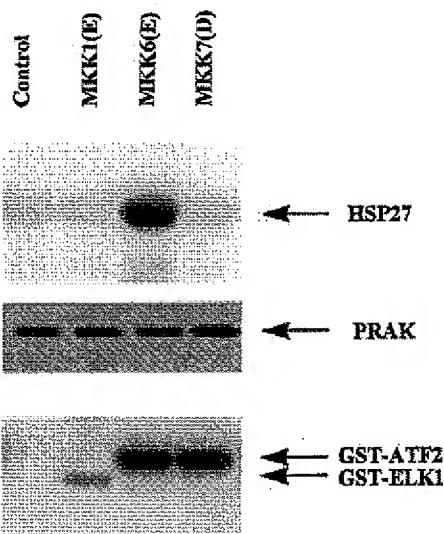


Fig. 9. Effect of individual MAP kinase pathways on PRAK activity in intact cells. The constitutively active form of MAP kinase kinase, MKK1(E), MKK6(E) or MKK7(D) was expressed in HeLa cells ($\sim 10^6$ per sample) using an adenovirus-mediated gene delivery system. The control infection was done with an adenovirus encoding β -galactosidase. An m.o.i. of 10 was used for each of the recombinant viruses, because nearly 100% infection efficiency was achieved at this titer. After 24 h, the cells were lysed and PRAK was immunoprecipitated using an anti-PRAK antibody. The lysates subsequently were used to immunoprecipitate p38 α / β , ERK2, p38 α / β or JNK1 (corresponding to the lanes in the figure) from the cells expressing β -galactosidase, MKK1(E), MKK6(E) or MKK7(D), respectively. The upper panel shows endogenous PRAK activity from different cell lysates measured by immunokinase assays using HSP27 as the substrate. The middle panel shows that equal amounts of PRAK determined by Western blotting were used for the immunoprecipitation. The lower panel shows endogenous activities of p38 α / β , ERK2, p38 α / β or JNK2 (corresponding to the lanes in figure) of the cells infected by the control virus, ad-MKK1(E), ad-MKK6(E) or ad-MKK7(D). GST-ELK1 was used as the ERK substrate, GST-ATF2 was used as the substrate for p38 and JNK. Comparable results were obtained in duplicate experiments.

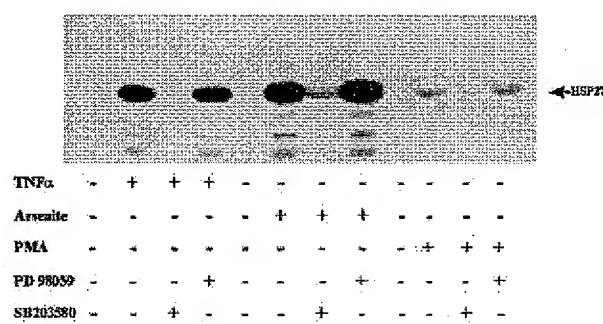
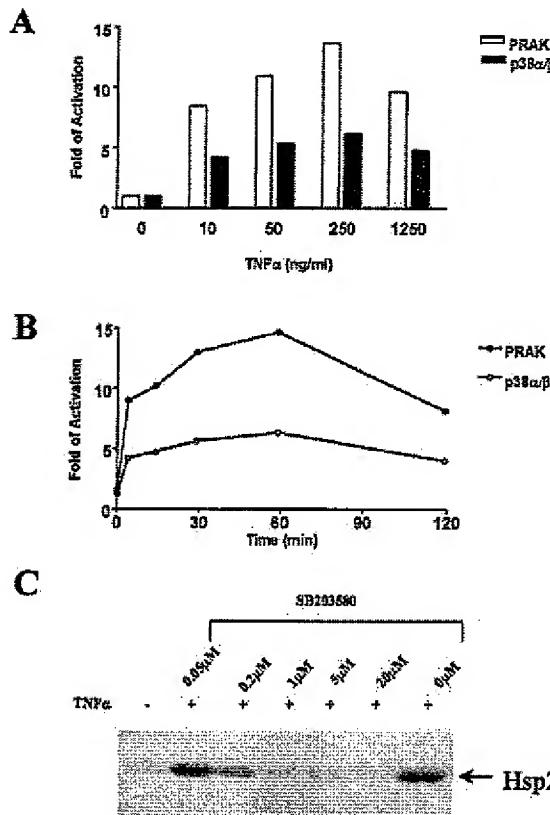


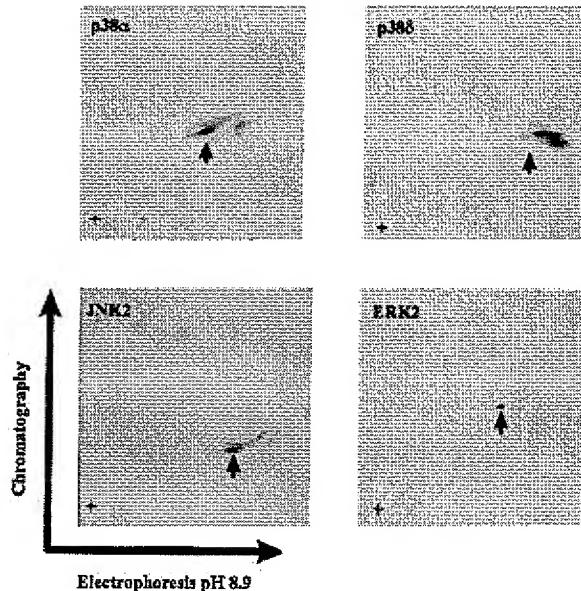
Fig. 10. Effects of SB203580 and PD98059 on endogenous PRAK activity in the cells treated with TNF- α , arsenite or PMA. HeLa cells ($\sim 10^6$ per sample) were pre-treated with PD98059 (10 μ M) or SB203580 (10 μ M) for 30 min, and then stimulated with TNF- α (100 ng/ml), arsenite (200 μ M) or PMA (100 nM) for 20 min. PRAK activities from these different treatments were measured by immunokinase assays with HSP27 as the substrate. The + or – under each lane indicates the presence or absence of a specific treatment. Comparable results were obtained in three experiments.

treated with a specific inhibitor for p38 α / β , SB203580 (Figure 10). Blocking of p38 α / β signaling dramatically inhibited arsenite- and TNF- α -, as well as PMA-induced



PRAK activation. In contrast, treatment of cells with PD98059 at a concentration (10 μ M) that inhibits PMA-induced ERK activation by 50% in HeLa cells (data not shown) had no effect on stress-induced PRAK activation, indicating that the ERK pathways does not play a role in regulating PRAK activity in HeLa cells. Taken together, these data indicate that p38 α and p38 β are *in vivo* regulators of PRAK.

To evaluate further the role of p38 α and p38 β in stress-induced PRAK activation, a series of experiments was performed to determine if p38 activation correlated with PRAK activation. HeLa cells were stimulated with increasing doses of TNF- α for 20 min or treated with 50 ng/ml of TNF- α for different periods of time. The activities of PRAK and p38 α / β were measured by immunokinase assays. As shown in Figure 11A and B, there is a good correlation between TNF- α -induced activation of both PRAK and p38. Moreover, TNF-induced PRAK activation was inhibited in a dose-dependent manner by the p38 α / β inhibitor SB20385, with an IC_{50} of 0.5 μ M (Figure 11C). A similar IC_{50} for arsenite-induced PRAK activation was found (data not shown). These data also suggest that PRAK and p38 are in the same signal transduction pathway.



Since no antibody is available to precipitate p38 α and p38 β differentially, we were unable to address their independent roles in PRAK activation in intact cells.

PRAK is regulated by the phosphorylation on Thr182

To analyze the phosphorylation site of PRAK by MAP kinases, phosphopeptide mapping was performed. Although differences were observed in the extent of phosphorylation of PRAK by p38 α , JNK2 and ERK2 (Figure 8A), the phosphopeptide maps of PRAK phosphorylated by these three MAP kinases appeared very similar (Figure 12). By calculating the R_f of the major phosphorylated peptides, we concluded that p38 α , JNK2 and ERK2 preferentially phosphorylated the same site in PRAK. p38 δ strongly phosphorylated PRAK but had no significant effect on PRAK activity (Figure 8). Phosphopeptide mapping of p38 δ -phosphorylated PRAK demonstrated that p38 δ phosphorylated a different site(s) in PRAK than p38 α . We conclude that p38 δ did not phosphorylate the regulatory site of PRAK.

PRAK was identified because it contained a conserved LMTP site in the T-loop. We suspected that this site was the regulatory phosphorylation site. To determine if the LMTP site was phosphorylated by p38 α / β , we prepared recombinant PRAK protein in which the LMTP sequence was mutated to LMDP [PRAK(182D)] and LMAP [PRAK(182A)]. Wild-type PRAK, PRAK(182D) and PRAK(182A) were treated with p38 β in the presence of [γ -³²P]ATP. ³²P-Labeled PRAK (wt), PRAK(182D) and PRAK(182A) were isolated and analyzed by peptide

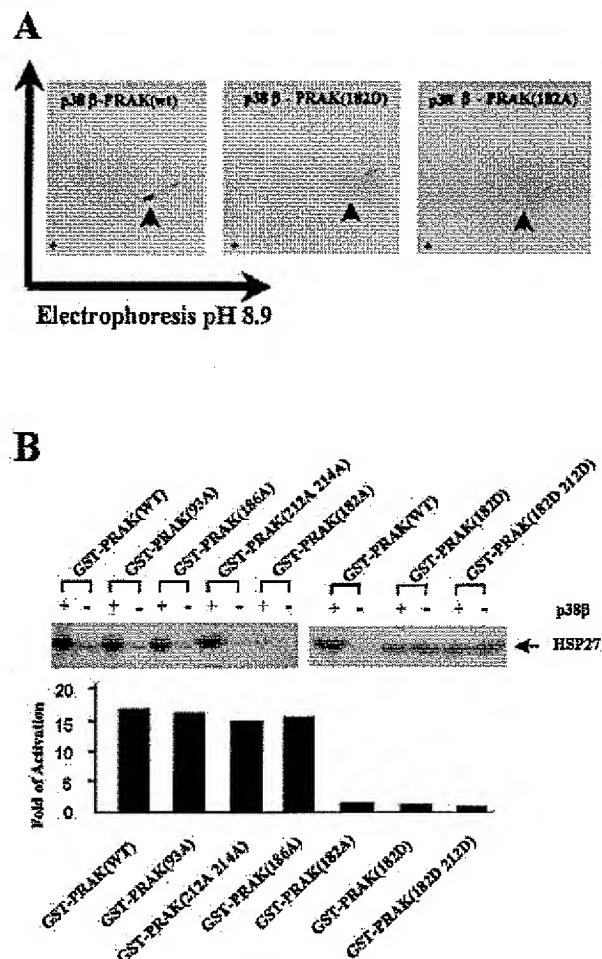


Fig. 13. Determination of the regulatory phosphorylation site of PRAK. (A) Wild-type GST-PRAK (wt) and two GST-PRAK mutants, PRAK (182A) and PRAK (182D), were treated with p38 β *in vitro* and subjected to phosphopeptide mapping using the same experimental conditions as in Figure 12. The small arrows inside each panel indicate the position of the PRAK peptide containing the putative regulatory phosphorylation site. This phosphorylation site disappeared when Thr182 was mutated to alanine or aspartic acid. (B) Each of the proline-directed serine or threonine residues in PRAK was substituted with alanine, and some of them also were converted to aspartic acid. The recombinant GST fusion proteins of these mutants were prepared. A coupled kinase assay was performed as described in Figure 8C using 10 μ g of HSP27 as the substrate and 2 μ g of each mutant or wild-type PRAK in the presence (+) or absence (-) of 0.2 μ g of p38 β . Kinase activities of GST-PRAK (wt), GST-PRAK (93A), GST-PRAK (182A), GST-PRAK (186A), GST-PRAK (212A, 214A), GST-PRAK (182D) and GST-PRAK (182D, 212D) were determined by quantifying the radioactive intensity of phosphorylated HSP27. The fold activation was calculated as described in Figure 8C and is shown as a bar graph. Mutation of Thr182 abolished activation of PRAK by p38 β while the other potential proline-directed phosphorylation sites had no role in PRAK activation. Comparable results were obtained in three experiments.

mapping. As shown in Figure 13A, p38 β phosphorylated the same peptide as p38 α (Figure 12). Mutation of Thr182 abolished the major phosphorylation site of PRAK. Edman degradation of this major phosphopeptide released free phosphate at the 8th cycle (data not shown), which is consistent with the position of Thr182 in the tryptic peptide. There are five proline-directed serine or threonine residues in PRAK that could be the sites for MAP kinase

phosphorylation. To confirm that Thr182 is the only regulatory site of PRAK, we created additional mutants that had Ser93, Thr186, Ser212 and Thr214 changed to Ala to prevent phosphorylation at each of these sites. The activity of wild-type or mutated PRAK proteins, before and after p38 α or p38 β phosphorylation, was determined by *in vitro* kinase assay. As shown in the left panel of Figure 13B, all mutants except PRAK (182A) were activated by p38 β to a similar extent, suggesting that the phosphorylation of these sites is not required for PRAK activation. Similar results were obtained with p38 α (data not shown). Mutation of Thr182 to Ala completely abolished the induction of PRAK by p38 β , demonstrating that Thr182 is indeed the regulatory site. Moreover, changing Thr182 to a negatively charged aspartic acid partially mimicked phosphorylation of PRAK, the mutant having ~50% of the fully activated PRAK activity (Figure 13B, right panel). Since there is a weak phosphorylated peptide that was phosphorylated by p38 β (Figure 13A), we sought to address whether this phosphorylation has any effect on PRAK activity. We have determined that the phosphorylation site is a serine residue by phosphoamino acid analysis and determined that it is Ser 212 by phosphopeptide mapping of PRAK(93A) and PRAK(212A, 214A) (data not shown). Since mutation of Ser212 to Ala does not have any effect on PRAK activation by p38 (Figure 13B, left panel), it is unlikely that phosphorylation at this site has a positive effect on PRAK activity. This was confirmed further by double mutation of Thr182 and Ser212 to aspartic acid. The additional aspartic acid mutation at Ser212 does not result in an enhancement of PRAK(182D) activity, supporting the idea that residue 182 is the sole regulation site of PRAK.

Discussion

PRAK is a new class of MAP kinase-regulated protein kinase and is regulated specifically by the p38 pathway

We have identified a new serine/threonine kinase, termed PRAK, which is strictly regulated by p38 α / β *in vitro* and *in vivo*. Like all known MAP kinase-regulated protein kinases, PRAK has a MAP kinase phosphorylation site (Ψ X[S/T]P) located in the T-loop between kinase subdomain VII and VIII. The overall sequence identity of PRAK to the known MAP kinase-regulated protein kinases is ~20–30%, which suggests that PRAK does not belong to any known group of MAP kinase-regulated protein kinases. We suggest that PRAK may represent a new protein kinase regulated by MAP kinases.

In addition to the relatively low homology of PRAK to the other known MAP kinase-regulated protein kinases, PRAK has structural and functional characteristics distinct from each of the known MAP kinase-regulated protein kinase groups. Structurally, PRAK is different from RSK members in that it lacks a second N-terminal kinase domain (Blenis, 1993). PRAK is regulated specifically by p38 α and p38 β , while RSKs are believed to be regulated strictly by the ERK pathway (Zhao *et al.*, 1996). The difference in downstream target(s) between PRAK and RSKs suggests that activation of RSKs and PRAK leads to different biological effects (Blenis, 1993). PRAK is distinct from MNK1/2 because MNKs are able to interact

with p38 α as well as ERK1/2 (Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997). MNK can phosphorylate eIF-4E while PRAK cannot, suggesting a difference in substrate specificity and biological function (Waskiewicz *et al.*, 1997). MAPKAP-K2 is probably the most closely related kinase to PRAK in terms of regulation and substrate specificity (Rouse *et al.*, 1994). Several features differentiate PRAK from MAPKAP-K2 and its isoforms. First, although PRAK and MAPKAP-K2 share a common feature, namely a regulatory phosphorylation site LXTP, the other phosphorylation sites found in MAPKAP-K2 are not conserved in PRAK (see protein sequence alignment shown in Figure 2). Since the multiple phosphorylation characteristic of MAPKAP-K (Ben-Levy *et al.*, 1995; Ludwig *et al.*, 1996) does not exist in PRAK, the structural basis for full activation of these kinase is probably different. Secondly, *in vitro* ERK is an equally potent activator of MAPKAP-K2 as p38 α (Stokoe *et al.*, 1992a; Ben-Levy *et al.*, 1995; Ludwig *et al.*, 1996) while PRAK is activated selectively by p38 α/β . This suggests that different structural features of PRAK and MAPKAP-K2 are required for upstream activator recognition. PRAK and MAPKAP-K2 appear to have a similar *in vivo* activation profile (Rouse *et al.*, 1994), but the mechanism of activation may be different. The inability of ERK to activate MAPKAP-K2 *in vivo* is likely to be due to the N-terminal proline-rich domain of MAPKAP-K2, which suppresses activating phosphorylation by ERK but not by p38 (Ben-Levy *et al.*, 1995). Since PRAK does not contain a proline-rich domain, the selectivity for p38 *in vivo* and *in vitro* may be determined by a different mechanism.

PRAK is a potential HSP27 kinase

HSP27 is phosphorylated in response to stimulation by cytokines, growth factors and chemical/physical stress. Two proteins, with molecular mass of 45 and 54 kDa, were found to be major HSP27 kinases in hamster and human cells (Huot *et al.*, 1995, 1997). Based on the (re)activation *in vitro* by ERK, antigenic properties and substrate specificity, at least one of the HSP27 kinases was believed to be MAPKAP-K2 or one of its isoforms (Huot *et al.*, 1995). The data presented here suggest that there are four major kinases that can phosphorylate HSP27 in HeLa cell lysates. Two of these kinases, with molecular masses of ~45 and 54 kDa respectively, are activated by the stress stimulus arsenite (Figure 5). It is unclear whether these two proteins correspond to the reported major HSP27 kinase activities in hamster CCL39 cells and human umbilical vein endothelial cells (HUEVCs) (Huot *et al.*, 1995, 1997). Our data demonstrated that PRAK is one of the inducible HSP27 kinases in HeLa cells. The following evidence is consistent with the idea that PRAK may be a physiological HSP27 kinase: (i) PRAK phosphorylates HSP27 at its physiologically relevant sites; (ii) PRAK activation is correlated with the activation of p38 α which has been shown to be correlated with HSP27 phosphorylation in response to stress (Huot *et al.*, 1997); (iii) activation of PRAK in intact cells is blocked by SB203580 which also blocks HSP27 phosphorylation (Guay *et al.*, 1997); and (iv) depletion of PRAK from stress-stimulated HeLa cell lysates removed the 54 kDa HSP27 kinase. MAPKAP-K2 has been reported to migrate on SDS-PAGE at a quite different molecular mass and often with

multiple bands depending on the species (Cano *et al.*, 1996). However, we only detected a 45 kDa protein in HeLa cell lysates using an anti-MAPKAP-K2-specific antibody (Figure 5). Since the PRAK antibody we generated cannot detect and immunoprecipitate recombinant MAPKAP-K2 protein (data not shown) and did not deplete the 45 kDa HSP27 kinase corresponding to MAPKAP-K2 (Figure 5), the 54 kDa HSP27 kinase in HeLa cells is PRAK. Further study is needed to address the relationship between PRAK and the previously identified major HSP27 kinases. MAPKAP-K2 was identified as a major HSP25 kinase because it co-purified with HSP25 kinase activity in rabbit skeletal muscle throughout its purification. It is not known whether PRAK co-purifies with the major HSP25 kinase or not. If not, it is possible that PRAK is a minor HSP27 kinase activity in cell extracts, but appears as a more major activity using *in-gel* kinase assays because it can be reactivated from SDS denaturation more readily than MAPKAP-K2. Whether PRAK is a physiologically relevant kinase for small heat shock proteins awaits further investigation.

The function of PRAK in the p38 pathway

The p38 MAP kinase pathway has been implicated in many biological process such as stress- and growth stimuli-induced gene expression (Lee *et al.*, 1994; Perregaux *et al.*, 1995), cytoskeletal reorganization (Huot *et al.*, 1997; Zechner *et al.*, 1997), cell proliferation and apoptosis (Xia *et al.*, 1995; Kawasaki *et al.*, 1997). The biological consequences of these responses are associated with many pathological changes that occur in the course of inflammatory/immunologic and cardiovascular diseases such as septic shock and cardiac hypertrophy (Bogoyevitch *et al.*, 1996; Yin *et al.*, 1997; Zechner *et al.*, 1997). As a downstream kinase, PRAK should amplify and diversifying p38's signals and thereby participate in one or more p38-mediated cellular responses. Since PRAK can phosphorylate HSP27 *in vitro* and phosphorylation of HSP27 has been suggested to play a role in actin reorganization, PRAK may mediate the downstream signal of the p38 pathway in regulating cytoskeletal organization. Two lines of evidence indicate that the p38 pathway plays an important role in regulating cytoskeletal organization: (i) activation of the p38 pathway is sufficient to confer sarcomeric organization in cardiac myocytes, and inhibition of p38 α/β by SB203580 blocked phenylephrine, an α_1 -adrenergic receptor agonist, induced sarcomeric unit formation (Zechner *et al.*, 1997); and (ii) in HUEVCs, oxidative stress-induced stress fiber formation was inhibited by SB203580 treatment (Huot *et al.*, 1997). In addition, overexpression of an inactive PRAK mutant in cardiac myocytes blocked p38 activation-induced hypertrophy characterized by sarcomeric unit formation and cell enlargement. However, expression of a constitutively active mutant of PRAK did not confer morphological changes observed by activation of the p38 pathway in cultured cardiac myocytes (Y. Wang, personal communication). Thus, PRAK may contribute to p38-mediated cytoskeletal reorganization in cardiac myocytes. Whether PRAK operates through phosphorylation of HSP27 to mediate p38 induced cytoskeletal changes awaits further investigation. Identification of other physiologically relevant

ant substrate(s) for PRAK is clearly the next important step in understanding the function of PRAK activation.

In summary, we have identified a new protein kinase regulated by the p38 MAP kinase pathway. The newly identified PRAK may represent a new group of MAP kinase-regulated protein kinases comparable with the RSK, MAPKAP-K and MNK groups. We have demonstrated that PRAK is strictly regulated by p38 α/β *in vitro* and *in vivo*. PRAK is a major stress-activated kinase in HeLa cells that can phosphorylate HSP27. Identification of PRAK provides more information regarding the biological function of the p38 signaling pathway. The biological consequence of PRAK activation deserves further investigation.

Materials and methods

cDNA cloning

Two peptide sequences, LXTPCYTPYYAP and LXTPCTANFVAP from MAPKAP-K2/3 and RSK2/3, were used to search for new homologies of human MAP kinase-regulated protein kinases in the dbest DDBJ/EMBL/GenBank database using the tblastn program. Clones containing the LXTP sequence flanked by unknown sequences were considered as novel clones. Seven clones with DDBJ/EMBL/GenBank accession Nos AA08236, W24753, AA08397, AA157440, R68329, N57069 and H09985 were found by this search encoding three different cDNAs. Clones, AA157440, R68392 and N57096 were purchased from Research Genetics (Huntsville, AL), and were analyzed further by complete DNA sequencing (The Scripps Research Institute core facility, La Jolla, CA). A total of 5×10^5 phages of a human placental cDNA library (Stratagene, La Jolla, CA) were screened using R68392 DNA as probe. Three positive phage clones were purified after the secondary screen and used for phagemid rescue. The three isolated plasmids contained inserts of 1.2, 1.6 and 2.0 kb. DNA sequencing of each clone indicated that all three clones were from the same gene. The clone containing the longest insert (containing the full-length PRAK cDNA) was sequenced completely on both strands.

5'-RACE

The MarathonTM cDNA amplification kit (ClonTech, San Francisco, CA) and Marathon-ready human placental cDNA (ClonTech) were used for 5'-RACE as described by the manufacturer.

cDNA constructs and expression plasmids

Four PRAK single mutants, PRAK (93A), PRAK (182D), PRAK (182A) and PRAK (186A), and two PRAK double mutants, PRAK (212A, 214A) and PRAK (182D, 212D), were created by substituting S93 with A, T182 with D and A, T186 with A, both S212 and T214 with A and both T182 and S212 with D in the PRAK cDNA using a PCR-based method (Innis *et al.*, 1990). Oligonucleotide sequences used in the PCRs are available upon request. Full-length PCR products containing the different mutations, wild-type PRAK and MAPKAP-K2 (Zu *et al.*, 1994) were cloned downstream and in-frame to glutathione S-transferase (GST) in the vector pGEX-KG (Guan and Dixon, 1991). Each mutant clone was characterized by DNA sequencing.

Bacterial expression constructs of His₆-tagged MKK6(E), MKK7(D), p38 α , p38 β , p38 γ , p38 δ , ERK2, JNK2 and HSP27 were constructed as described previously (Jiang *et al.*, 1996, 1997). The spliced form of p38 β with 364 amino acids (also known as p38 β_2 DDBJ/EMBL/GenBank # AF001008) was used in the experiments described in this study. The HA epitope-tagged wild-type PRAK and mutant PRAK (182D) were cloned into the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA) by a PCR recombination method as described (Jiang *et al.*, 1996).

Recombinant proteins

GST fusion proteins of ATF2 (1–109) (Han *et al.*, 1996), c-Jun (1–93) (Derijard *et al.*, 1994), ELK1 (307–428) (Marais *et al.*, 1993) and those described above were expressed in the bacterial strain BL21 and purified using glutathione-Sepharose 4B beads (Pharmacia Biotech, Uppsala, Sweden) as described by the manufacturer. All of the His₆-tagged recombinant proteins were expressed in the BL21(DE3) strain and purified using the Ni-NTA purification system (Qiagen). PHAS-I was

purchased from Stratagene (La Jolla, CA). Myelin basic protein (MBP) was from Sigma (St. Louis, MO). The eIF-4E expression plasmid was obtained from Dr C.G.Proud (University of Kent at Canterbury) and the recombinant eIF-4E was purified as described (Edery *et al.*, 1988). Activated MEK1 protein was a gift from Dr Kunliang Guan (University of Michigan Medical School).

Full activation of recombinant p38 α , p38 β , p38 γ or p38 δ *in vitro* was achieved by incubation with recombinant MKK6(E) at a 5:1 molar ratio at 37°C for 15 min in the presence of ATP. Full activation of recombinant ERK2 and JNK2 was achieved by incubation with MEK1(E) or MKK7(D) respectively. The different dose of MKKs and time of incubation have been tested to optimize the conditions for full activation of these recombinant MAPKs (data not shown). PRAK or MAPKAP-K2 were activated by incubating GST-PRAK or GST-MAPKAP-K2 fusion proteins coupled to glutathione-Sepharose beads with p38 α and ATP at 37°C for 40 min with shaking. After incubation, the beads were washed three times with phosphate-buffered saline (PBS) to remove p38 α and free ATP. Activated GST fusion proteins were then eluted as described above.

Northern blot analysis

A multiple human tissue mRNA blot containing 2 μ g of poly(A)⁺ RNA from various tissues was purchased from Clontech (San Francisco, CA). The blot was hybridized as described (Jiang *et al.*, 1996) to a probe prepared by labeling the coding region of the PRAK cDNA fragment with [α -³²P]dCTP using random priming (Han *et al.*, 1991).

Preparation of anti-PRAK antibody

Two rabbits were used to generate anti-PRAK polyclonal antibody using recombinant PRAK protein as described (Ausubel *et al.*, 1995). The specificity of the antibodies was examined by Western blot analysis and by immunoprecipitation using recombinant PRAK and MAPKAP-K2. The antibody raised in both rabbits can specifically detect PRAK, but not MAPKAP-K2 (data not shown). No detectable MAPKAP-K2 can be precipitated by anti-PRAK antibodies (data not shown).

Western blot analysis

Total cell lysates were prepared using lysis buffer A: 20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 10% glycerol, 1 mM Na₃VO₄, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100. Equal protein loading of cell extracts in SDS-PAGE was determined by Bio-Rad protein assay solution (Bio-Rad, Hercules, CA) and by staining the transferred nitrocellulose membrane with Ponceau S solution (Sigma, St Louis, MO). Rabbit polyclonal antibodies raised against bacterially expressed recombinant GST-PRAK and goat polyclonal antibody against MAPKAP-K2-specific peptide (RVDYEQIKKKIEDASN) (Santa Cruz Biotech, Santa Cruz, CA) were used for Western blots. Standard Western blot methods were used (Han *et al.*, 1993).

Protein kinase assays

In vitro kinase assays were performed using standard experimental conditions as described (Jiang *et al.*, 1996). Prior to MAP kinase and MAP kinase-regulated protein kinase-coupled assays, the recombinant MAP kinases were activated by bacterially expressed constitutively active MKKs as described above. Kinase assays were carried out at 37°C for 30 min in a reaction volume of 40 μ l, containing equal amount of each MAP kinase (0.5 μ g) determined by Coomassie Blue staining, ~2 μ g of GST-PRAK or GST-MAPKAP-K2, 10 μ g of substrate, 250 μ M ATP and 10 μ Ci of [γ -³²P]ATP. Reactions were terminated by addition of Laemmli sample buffer. Reaction products were resolved by SDS-PAGE and the extent of protein phosphorylation was visualized by autoradiography or phosphoimaging.

For the kinase assays with immunoprecipitated endogenous protein kinase or transiently expressed HA-tagged protein, ~10⁶ cells grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) were used for each sample. The cells, with or without pre-treatment with SB203580 (Calbiochem, San Diego, CA) or PD98059 (New England Biolabs, Beverly, MA), were treated with different stimuli for the times specified in the figure legends or text. A total of 10⁶ cells were lysed using 300 μ l of lysis buffer containing 1% Triton X-100 as described (Jiang *et al.*, 1996). Endogenous PRAK or transiently expressed HA-tagged PRAK was precipitated by adding 10 μ l of an anti-PRAK antibody or 2 μ g of anti-HA monoclonal antibody 12CA5 to the nuclei-free cell lysates respectively and incubating for 2 h at 4°C, and then adding 10 μ l of protein G-agarose beads and incubating with moderate stirring at 4°C for a further 1 h. The beads were washed five times with 1 ml of lysis buffer and subsequently used for kinase

assays using HSP27 as a substrate. p38 α/β were precipitated by antibody against recombinant p38 α which has cross-reactivity to p38 β . ERK2 and JNK1 were immunoprecipitated using anti-ERK2 (UBI) and anti-JNK1 polyclonal antibodies (obtained from Dr M.Karin, University of California at San Diego) respectively. GST-ELK1 was used as substrate for ERK2. GST-ATF2 was used for JNK2 and p38 α/β .

In-gel kinase assay

Ten μ g of HeLa cell lysate with or without arsenite treatment was resolved by SDS-PAGE using a gel polymerized with 0.5 mg/ml HSP27. The separated proteins were denatured and renatured as described (Wang and Erikson, 1992). The kinase reaction was performed in 5 ml of the kinase buffer described above containing 50 μ M ATP and 50 μ Ci of [γ - 32 P]ATP for 1 h at 30°C. The reaction was terminated by washing the gel with a fixing solution containing 10 mM sodium pyrophosphate and 5% trichloroacetic acid. The gel was dried and subjected to autoradiography.

In vitro binding of PRAK and p38

GST-PRAK bound to glutathione-agarose beads and His-tagged p38 α , p38 β , p38 γ , p38 δ , ERK2 and JNK2 were used to examine the *in vitro* interaction of PRAK with each of these MAP kinases as described (Han *et al.*, 1997).

Construction and preparation of recombinant adenovirus

The recombinant adenovirus encoding MKK1(E) [also known as MEK1(E) or MKK6(E)] was constructed and prepared as previously described (Huang *et al.*, 1997b). The spliced form of MKK6 with 334 amino acids (also known as MKK6b) was used in the experiment. MKK7D expression virus was created using the same method (Huang *et al.*, 1997b). We established that nearly 100% infection of HeLa cells can be achieved at an m.o.i. of 10.

Phosphoamino acid analysis, phosphopeptide mapping and Edman degradation

These experiments were performed as described by Boyle *et al.* (1991).

Cell transfections

HeLa cells were plated in 6-well plates with 50% confluence in DMEM supplemented with 10% FBS. After 24 h, the cells were transfected with expression vectors containing HA-tagged PRAK or empty vector pcDNA3 using lipofectamine (Life Technologies, Inc., Grand Island, NY). After 36 h, the cells were treated with the different stimuli for 30 min and the kinase activity of HA-tagged PRAK was assayed as described (Han *et al.*, 1996).

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